FACSymphony A5
USER MANUAL

If you have any question concerning operating and maintaining the Symphony, please contact the core facility staff.

The BD FACS DiVa Software is described briefly in the BD FACS DiVa Software Quick Guide.

- Booking appointment for the Symphony
- Starting the Symphony
- Quick clean procedure
- Daily shutdown procedure
- Removing air from system
- Symphony: Differences from LSR II/Fortessa
- Controls and compensation for Flow Cytometry analysis
- Using the Symphony with the HTS (plate loader)

BOOKING

Online booking: www.icr-booking.no

Please contact the core facility staff to receive a personal username and password for the booking.

Very important!

Before you leave the Symphony, contact the next user on the list to confirm the booking. If it`s more than **1 hour** until the next user is coming, please turn off the instrument after cleaning. However, if the next user cannot be reached you have to turn off the instrument. Please contact us if this happens.

STARTING THE INSTRUMENT

For use of the Symphony with HTS (plate reader), please see the last pages.

1. If needed; put on a new box FACSFlow with surfactant **AND** empty the waste in the sink. Put the waste box back on the FFSS with the cut-out window towards you.

2. Start the computer and turn on the instrument
   - Username:
   - Password:

3. Start FACS DiVa Software:
   - Username:
   - No password needed

   ALWAYS PRESS "USE CST SETTINGS", when prompted!

4. Open the BD Coherent Connection 4 software (desktop). To start the UV laser: In the UV speedometer's bottom left corner, type in 60 mW, press ENTER, wait for the laser power to be activated (black letters, and typically showing 59,8 mW). Press "Laser START".

5. Run the prime procedure to remove air from the flow cell: Tube support arm in the sideways position. Remove tube docked onto the injection port. Press the PRIME-button at the fluidics control panel. **Repeat 2 times!**

6. Install a tube with 3 ml ddH$_2$O and leave the support arm to the side and let it run for 1 min at HI. Place the support arm under the tube and run the sample for further 5 min.

The UV laser needs 20 min pre-warming, the rest of the lasers <u>only 5 min</u>.
- THE MACHINE IS NOW READY TO USE -

7. Start CST from the Cytometer tab. Press RUN, LO, **Fine Adj 300** on the cytometer. Always prepare a fresh tube of CST beads: 1 drop CST beads (vortex black vial in the fridge) + 150 µl dH$_2$O. Run "Check Performance".

8. **If you don't need the UV laser in your experiment, turn it off**! Use the BD Coherent Connection 4 software (desktop). Press "Laser STOP" on the UV laser speedometer.

9. Remember to PRIME the Symphony at least once per hour, RUN dH$_2$O for a minimum of 2 minutes before running samples or CST after a PRIME.

If you leave the machine for more than 5 min, place a tube containing at least 1 ml of dH$_2$O and click STNBY.

10. Write your name, institute/department, date and instrument settings in the log book (in the cupboard above the screens).

11. When you are finished running your samples:

        A. Perform the daily shutdown procedure (see below)

                OR

        B. If you are certain the next user on the timetable is attending their session, perform the Quick Clean Procedure (see below).

QUICK CLEAN PROCEDURE

Install a tube containing 3 ml of clean solution (A). Leave the support arm to the side and let it run for 1 min with the flow rate set to HI. Move the support arm under the tube and allow the clean solution to run for 2 min with the flow rate set to HI.

Repeat the procedure with Rinse solution (B) and ddH$_2$O.

Important: If more than 1 hour until next booking TURN OFF the instrument.

Leave the instrument in STNBY with ddH$_2$O loaded to the sample injection port with the support arm underneath the tube.

The next user must make sure all the required lasers are ON and pre-warmed! One PRIME + 5 min run with ddH$_2$O on HI prepares the fluidics after a STNBY period.

CHECK THE SHEATH TANK AND THE WASTE BEFORE YOU START UP/ LEAVE THE INSTRUMENT. Boxes of FACSFlow with surfactant (Sheath fluid) are placed under the bench on the opposite side of the room, and the waste can be emptied in the sink. NB: Turn off the alarm during the exchange by pressing the ALARM button on the FFSS– and **always remember to press the RESTART after** the exchange is completed!

DAILY SHUTDOWN PROCEDURE

Performed by the last user of the day or when if the next user cannot be reached.

1. Label and fill three tubes with 3 ml of solution A(clean), solution B(rinse) and ddH$_2$O.

2. Install the 3 ml of clean solution (A) on the sample injection port with the support arm to the side and let in run for 1 min.

3. Move the support arm under the tube and allow the clean solution to run for 10 min with the flow rate set to HI.

4. Repeat step 2 and 3 with rinse solution (B).

5. Repeat step 2 with dH$_2$O and then run dH$_2$0 for 3 min at HI. Leave the tube in the sample injection port with the support arm underneath the tube. Set the machine on STNBY and flow rate at LO. It is now safe to turn off the Symphony. NB: The FFSS (fluidics cart) automatically shuts down with the instrument.

6. Close your experiment.

REMOVING AIR FROM THE SYSTEM

If the FACS Fluid Supply Cart is not turned ON or if the sheath fluid box runs empty, air is drawn into the system. Air in the system strongly affects the performance of the machine. Check if there is any sheath in the sheath reservoir beside the cytometers on the desk.

If there is air in the filter (behind the sheath reservoir on the desk), gently tap the filter body with your fingers to dislodge the bubbles and force them to the top. Open the valve while slightly flicking the filter to make sure that all the air has escaped through the valve (using the small waste beaker standing beside the filter).

To remove air from the flow cell, use the prime option several times.

If you have air in the system, please contact the core facility staff.

TRAINING

Novice users will be taught via hands-on training to acquire and analyze their own data. Contact the core facility staff to make an appointment. More experienced users will be able to use the Symphony alone, but are welcome to ask for technical expertise and advice for experimental planning and design, cell preparation, analysis and data handling.

DATA ANALYSIS

Data analysis training can be provided by the staff. The flow facility has a fast workstation with FlowJo software for users in room K03.066. No USB-stick/external harddisk is allowed on that computer either.

Automatic backup of your FCS files is done locally on the PC, the exact location is **D:\BDExport\FCS.**

Here the files are sorted under the date of analysis and then under your experiment's name, that is why it is important that you give your experiments a name that contains your name or initials.

Important! You still have to remove old files from the DiVa software and you are still responsible for your own backup.

Symphony: New features from LSR

1. **New flow cell plastic:** PRIME at least once per hour, RUN dH$_2$O for 2 minutes before running samples or CST after a PRIME. Sheath Fluid with surfactant reduces the problem with the air bubbles in the flow cell, but regular PRIMING is still essential.

2. **New electronics:** The number of parameters in the Symphony forced the development of new electronics. Consequences:
    I. Faster connection
    II. Less noise/background in the PMTs
    III. Wider measurement range (max scale 500.000)
    IV. Bi-exponential transformation does not perfectly match the new electronics.
        i. Autoscaling does not work well. Use manual scaling to reduce the negative display (Biexponential editor).
        ii. You may see your background signal split in two around zero. This is just an artefact of the biexponential display and not how your data is actually distributed.

3. **Laser power:** Our Symphony has higher laser power than most instruments. By default we are not using the full potential from each laser, apart from 638nm. If you have really dim markers we can test if increasing the laser power increases the sensitivity in your experiment.

4. **Less dead volume in tube mode:** The new biohazard pump decreases the dead volume when running in tube mode.

Oslo University Hospital

Controls for Multi-color Flow Acquisition

Isotype Controls: As always for flow cytometry, it is important to evaluate the specificity of your staining and determine whether isotype specific background binding is a problematic factor. With this in mind, it is important to realize that isotype controls are not the proper way to determine what the boundary for positive and negative expression of a marker in multi-color experiments.

Background (FMOC) Controls: In multi-color flow cytometry, the absolute background in a given detector is a function of all the colors that spill over and requires compensation. Extremely bright fluorochromes can also give unexpected spillover. Due to spillover and photon counting error in the PMTs the level of background in a corrected detector increase (Signal Spread Error=SE), this spread will limit the sensitivity in that detector. The proper way to evaluate the background is to have a control that is all the other stains that you will be using, except for the color detected in that channel, hence the name "fluorescence minus one control" (FMOC). This control is a must if you are trying to evaluate very dim populations in multi-color space.

	Unstained Control	FMO Control	Fully Stained
FITC	–	**CD3**	**CD3**
PE	–	–	**CD4**
Cy5PE	–	**CD8**	**CD8**
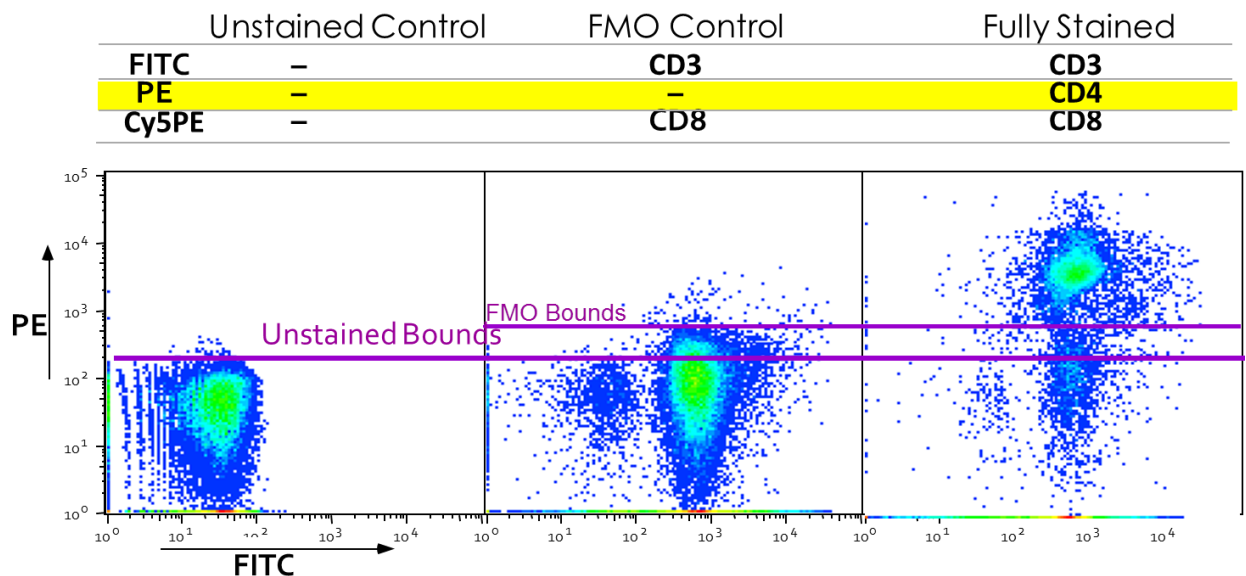
Slide Courtesy of Mario Roederer, Ph.D., NIH Vaccine Center

COMPENSATION:

Proper compensation is repeated (or at least checked) for every experiment. If the cell types differ, the reagents differ, or when any instrument settings change, a new compensation calculation MUST be performed. Compensation makes sure that the centers of the positive and negative cell populations are aligned by matching the median fluorescence intensity.
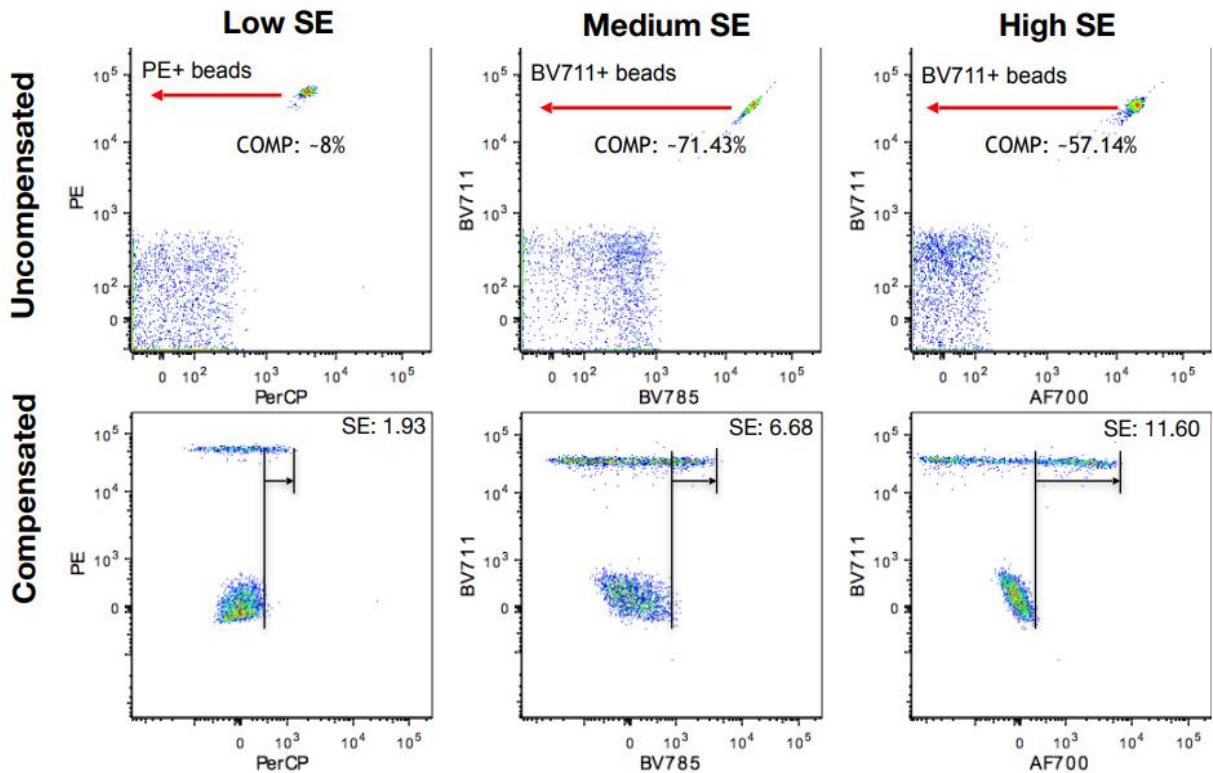
Compensation Controls:

1. A stained sample to determine appropriate PMTV for the best resolution of your dim vs. negative populations. Our suggested PMTV setting is a good starting point and resolution normally deteriorates below the suggested setting.
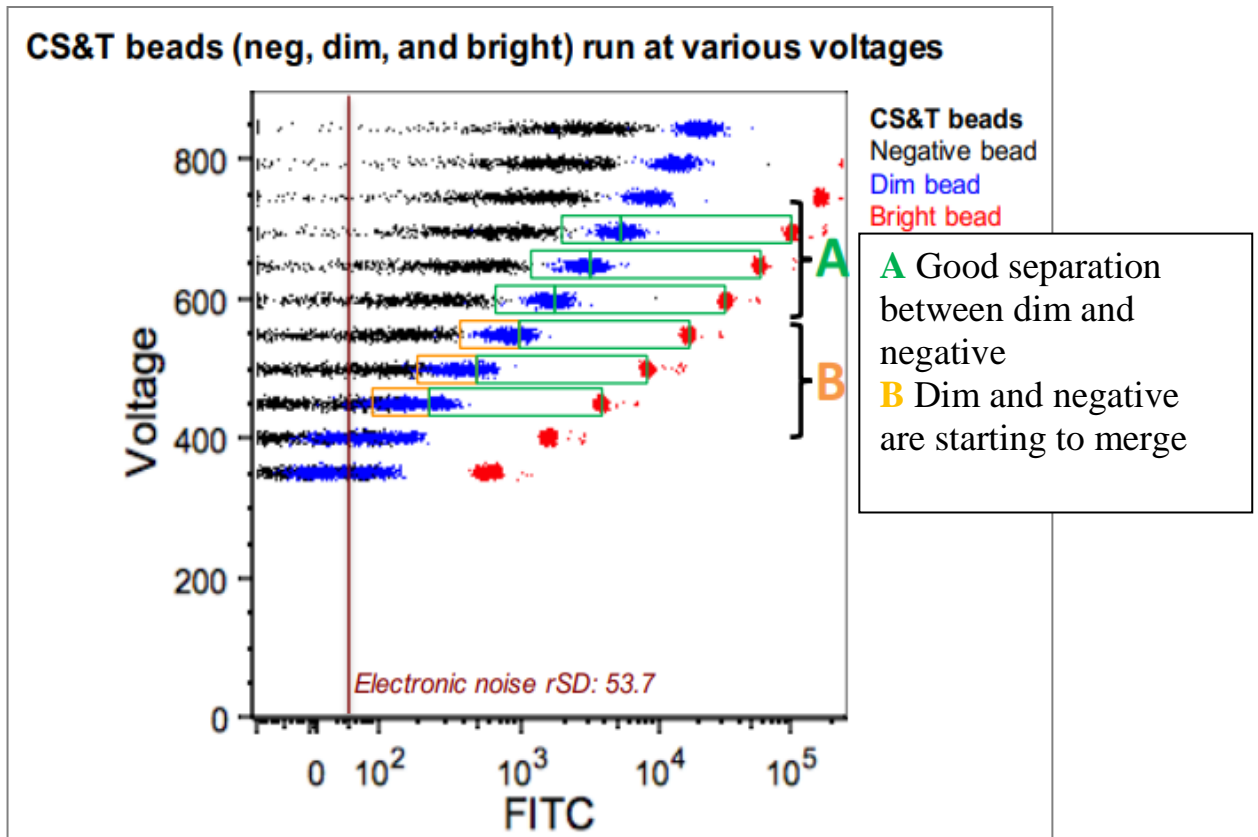
Adapted slide from *The impact of adjusting PMT voltages on spillover and compensation* by Thomas M. Ashhurst, Adrian L. Smith at Sydney Cytometry Facility, The University of Sydney

o Never reduce PMTV to reduce the comp. values! Compensation only reveals signal spread error (SE) it does not create it.

o If your comp. controls are out of the PMTs linear range with the optimal PMTV, you should make a weaker comp. control. Only reduce the voltage of your PMT if you see that this setting does not reduce the resolution in your sample.

Adapted slide from *The impact of adjusting PMT voltages on spillover and compensation* by Thomas M. Ashhurst, Adrian L. Smith at Sydney Cytometry Facility, The University of Sydney

2. Compensation controls (1 for each color) must adhere to these rules:

o The fluorochrome MUST be the exact same as in your sample. E.g. the spectrum of FITC does not match GFP. Additionally, if you are using tandem-conjugates (e.g. PE-Cy7, BV711) it must be the same lot of antibody.

- o **Background fluorescence MUST be matched between your positive and negative population!**
  - − If you use beads for positive controls, use unstained beads as negative control.
  - − If you use cells as positive controls, make sure you do not compensate against a different cell type in your negative control. E.g. Monocyte marker (positive) and lymphocytes (negative).
  - − In a setup where you need to use some controls with cells and some with beads, NEVER use the universal unstained. Make sure you have negative and positive population in each single stained control tube. FlowJo provides the possibility of switching between separate negative controls and internal negative controls for different markers.

✓ **Same background in negative and positive**

✗ **Not the same background in negative and positive**
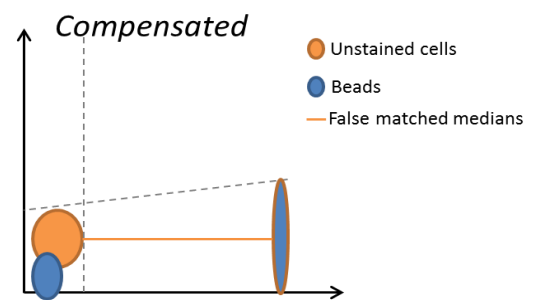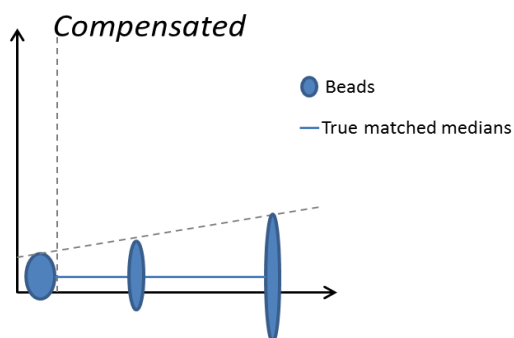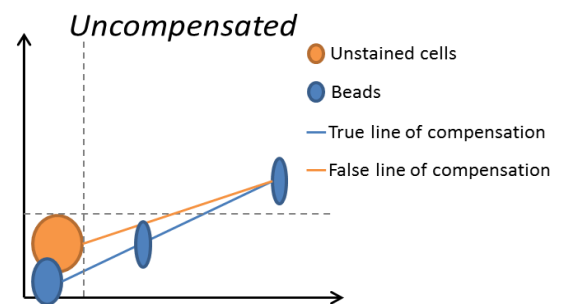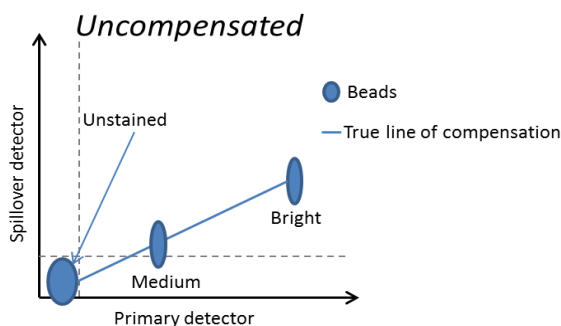
- o **Intensity MUST be at least as bright as your brightest sample** (a dimmer control may not properly compensate an extremely bright sample).
- o Feel free to increase the recorded events setting (15.000-30.000), especially if you have a small fraction of positive/negative events in any sample. The statistical power behind the calculation of compensation values will increase.

Using the HTS plate loader.

NB: Requires additional training by the Core Facility Staff!

(The BD HTS User guide is located in the cupboard above the instrument)
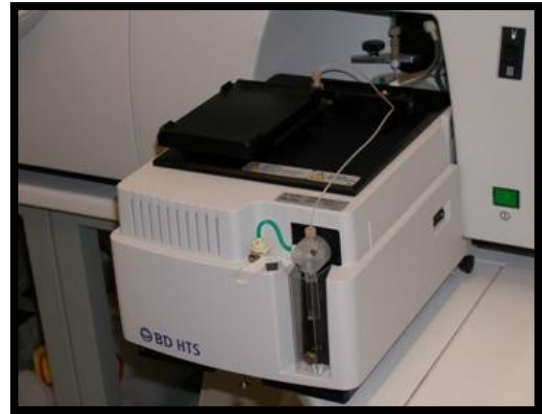
Plates compatible with the HTS:

96-well U bottom (Cat. No. 353910)

96-well V bottom (Cat. No. 353263)

96-well flat bottom (Cat. No. 353915)

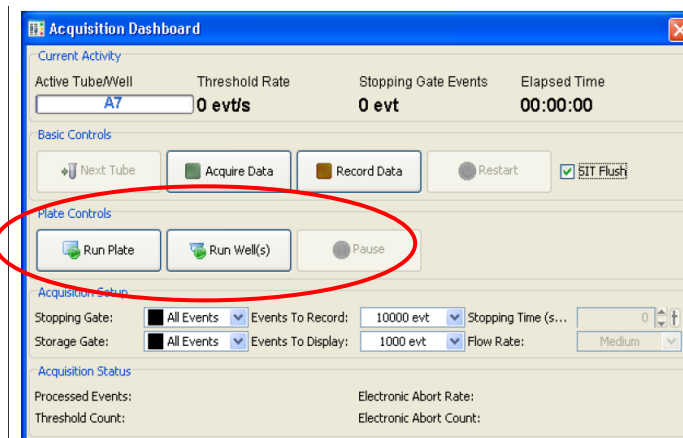384-well flat bottom (Cat. No. 353233)

Falcon is sold to Corning now, so the plates are only available from Corning and its distributors but they still have the same catalog number.

DO NOT USE PLATES FROM OTHER VENDOURS THAN FALCON/CORNING/COSTAR, TO PREVENT HARM TO THE HTS!

1. Make sure there is enough DI water in the 1 L glass bottle. This is the fluid going into the HTS. Refill with MilliQ water if necessary.
2. Always perform a CST "Check Performance" with a tube if you are starting up the instrument. Do not run CST from the HTS!
3. Switch the button on the right side of the instrument from "tube" to "plate" position.
4. Screw of the metal sleeve (SIT protector) around the sample injection needle. Replace it with the shorter HTS sleeve (keep the grey screwer), which is stored in water (refill if necessary) in a 15ml tube in the penholding-mug. Be careful not to bend the sample needle!

SIT protector

tightening nut

gap

Make sure there is a gap here!

sample coupler
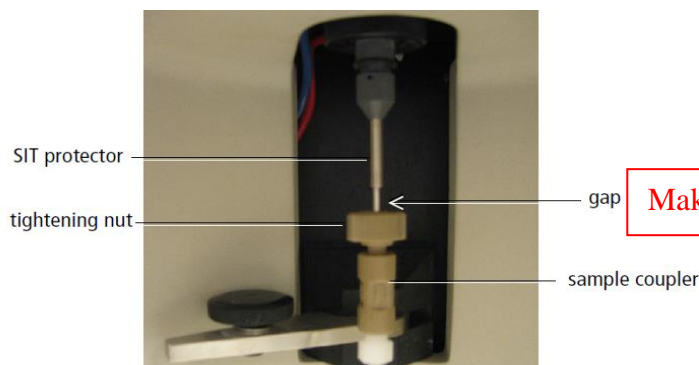
5. Press "RUN" and "HI", you cannot use the HTS if the instrument is in STNBY.

6. Run the Reinitialize procedure (from the HTS menu): the dialog "HTS Initialization complete" should come up

7. Prime the HTS (from the HTS menu) several times, until you see no more air in HTS tubings/syringes, and then you prime one more time ☺ (air in the orange waste tube is <u>not</u> a problem).

8. Right-click in a blank area on the Acquisition Dashboard (see under) to show the "Plate control"

- The HTS does not work unless the big plastic cover is placed correctly on top.

INSTITUTE FOR CANCER RESEARCH

New Reservation

Cancel ⊘ Create

Heidi Ødegaard Notø (heinot@rr-research.no) Change 👤

Begin	20/11/2020	11:00	⌄
End	20/11/2020	11:30	⌄

0 days 0 hours 30 minutes

Repeat Does Not Repeat ⌄

Resources Change ➕   **Accessories** Add ➕

LSR II UV laser

Title of reservation

Description of reservation

Samples in tubes or plate?

Plate (HTS) 🖱️

DISCONNECTING THE HTS

All HTS users should choose "Plate (HTS)" in in the drop-down menu "Samples in tubes or plate?" when creating their booking, in case the next user also wants to use the HTS and there is no need for disconnecting it. Proceed with the disconnection if you are not 100% sure, as most users do not know how to disconnect the HTS.

1. Wash the HTS using a washing plate (in the cupboard) filled with A Clean and dH$_2$O as described in the experiment "HTS Wash" under the folder Service

2. Make sure the next user is not also using the HTS before disconnecting it.

3. Switch the button on the instrument back to "tube" mode 🔘

4. Replace the HTS metal sleeve SIT Protector with the longer one used for running in tube mode. The HTS sleeve is to be stored in 15ml tube with water (refill if necessary).

5. Perform the instrument PRIME procedure (see point 4 in the HTS start up if you need more information).

6. Press STNBY, install the dH$_2$O tube (refill if necessary) and put the support arm under the tube.

7. Make sure the software layout (Dashboard, Inspector, Worksheet) is back to normal, and close the Plate window.

total volume — 250 µl

available volume — 200 µl

plate-dependent dead volume — 30 µl
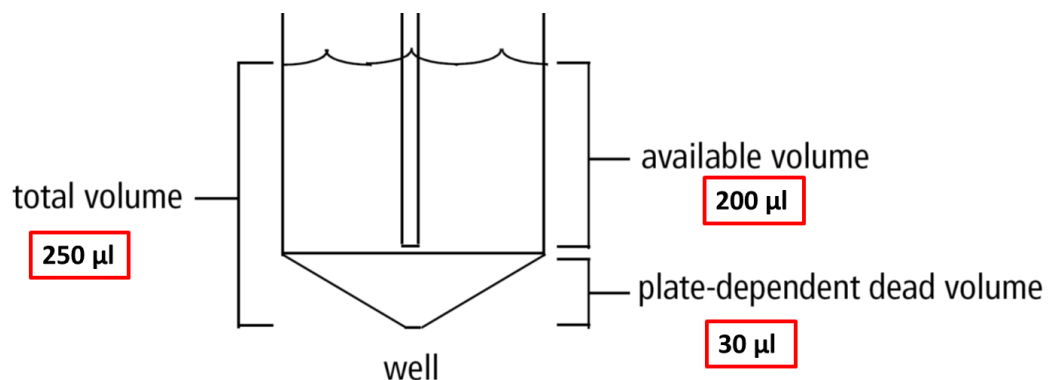
well

Available volume: Volume pipetted into well minus aspirated excess volume (**20µl**) and dead volume (**30 µl**)

Mixing volume: Approximately one-half the available volume. NB! A mixing volume that is larger than the available volume introduces air bubbles in the sample

Minimum volume: **50 µl**

Mixing speed (µl/sec): **180 (25-250)**

Number of mixes (cycles): **2 (0-5)**

Wash volume (µl): **400**